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A microbial sensor for detecting inhibitors of nitrification in wastewater

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Abstract

A biosensor for rapid and reproducible measurements of inhibitors of nitrification in environmental samples has been developed. The biosensor is mainly designed to be used for wastewater control and consists of a Clark oxygen probe as a transducer and an immobilised mixed nitrifying culture as the microbial component. The measuring principle is based on the direct determination of bacterial metabolic activity by measuring the oxygen consumption rate of the microbial immobilisate. Both the prototype of a laboratory device and a field device have been realised. The laboratory device can be used to determine the nitrification inhibiting effect of individual chemical compounds as well as of environmental samples. The field device was constructed for on-line monitoring of inhibitors in sewage systems.

Keywords: Microbial sensor; Nitrification; Inhibitors; Monitoring of wastewater; Sewage plants

1. Introduction

Removal of nitrogen is achieved in most wastewater treatment plants by biological processes involving nitrification and denitrification. It is well known that many industrial wastewaters occasionally contain toxicants that can inhibit these processes. Additionally, autotrophic nitrifiers are very sensitive to toxicant shock loads (Fearnside and Booker, 1995; Grüttner et al., 1994; Kroiss et al., 1992). Due to the slow growth of nitrifiers and their small percentage in the total biomass of the sludge, it is often very difficult or even impossible to keep the biological nitrification process efficient and thus meet prescribed effluent standards (Massone et al., 1996). Disturbances of nitrification in sewage plants can lead to high concentrations of ammonium or nitrite ions in the effluent, which both act as oxygen consuming and fish-toxic agents in surface waters.

Thus, there is a need for a fast and sensitive on-line detector of inhibiting substances. The detector should either be used at the influent side of the treatment plant as a warning device or directly in the wastewater channel to detect pollutants from industrial plants. Although a few biosensor systems for on-line monitoring of wastewater have already been described (Massone et al., 1996; Kong and Verstraete, 1993; Kong, 1995), there is still a need for commercially available mobile measuring devices capable of direct, rapid and reliable detection of toxic agents in situ (Weetall, 1996; Rogers, 1995).

Moreover, the currently employed laboratory methods for the quantitation of inhibiting effects of environmental samples are relatively complicated and require relatively high expenditures of time and man-power (Fearnside and Booker, 1995; Wagner and Kayser, 1990; ISO, 1989). Thus, the results of these tests are often obtained too late to react and take suitable measures in the sewage plant.

In this paper we describe a biosensor for detecting inhibitors of nitrification. This biosensor provides a solution for the problems characterised above. Microbial sensors generally offer several advantages for use in environmental analysis, because they are simple and cheap to manufacture and, as self-regenerating systems, can be used repeatedly (Karube et al., 1995; Rogers and

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Lin, 1992). Above all, microbial sensors exhibit a so-called multireceptor behaviour (Riedel et al., 1990), which allows a great variety of substances to be detected simultaneously with the same sensor. Microbial sensors are therefore advantageous for measuring environmentally relevant summary parameters or for detecting integral effects such as toxicity.

2. Experimental

2.1. Microorganisms

The microbial component of the sensor used in our experiments consisted of a mixed culture of nitrifiers which was enriched from a nitrifying sewage plant. The cultivation was carried out continuously in a chemostat. Due to a reactor volume (V) of 10 l and a nutrient solution feed (f) of 0.3 to 0.4 l h^{-1}, a dilution rate ($D = fV^{-1}$) of 0.03 to 0.04 h^{-1} was obtained.

The nutrient solution consisted of 200 mg l^{-1} urea, 1.2 g l^{-1} disodium hydrogen phosphate (Na_2HPO_4, anhydrous) and 1.0 g l^{-1} potassium dihydrogen phosphate (KH_2PO_4). These substances were diluted in water with a resulting pH of 7.8. The following trace elements which are essential for nitrifiers were added to the nutrient solution: 45 μg l^{-1} $MnSO_4$, 49 μg l^{-1} H_3BO_3, 43 μg l^{-1} $ZnSO_4 \cdot 7\,H_2O$, 16 μg l^{-1} $CuSO_4$ and 37 μg l^{-1} $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$. The enriched nitrifying mixed culture was characterized by GC-chromatograms of the membrane lipids (König et al., 1997a).

2.2. Immobilisation of the nitrifiers and assembly of the sensor

The immobilisation of the nitrifying culture is based on its inclusion in polycarbamoylsulfonate (PCS), a non-toxic polyurethane (SensLab, Leipzig, Germany). The immobilisation was described in previous publications (König et al., 1995, 1997a; Vorlop et al., 1992). Finally, the biosensor was manufactured by fixing a membrane on a Clark oxygen probe with a special clamp commonly used for the assembly of amperometric sensors.

2.3. Measuring system

For laboratory investigations a modified commercial device, ARAS (Dr. Bruno Lange GmbH, Berlin, Germany), was used as described in previous publications (Riedel et al., 1985). For on-line investigations with real wastewater, a sensor system based on the TriOxmatic 300 oxygen probe with the OXI 3000 measuring device (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) was employed.

This field device for on-line monitoring of wastewater directly in the sewage system consisted of a continuous flow injection analysis system (FIA) with the biosensor integrated in a special flow-through cell that was developed at our institute. Included also are several on-line measuring points for pH, O_2 and NH_4^+, which are necessary for monitoring the parameters vital to the immobilised nitrifying culture. The whole field device is completely controlled by a PC-system. The prototype was built up in a container with 3 m length, 2 m width and 2 m height.

To provide the sensor permanently with ultrafiltered wastewater directly from the wastewater channel, we used a wastewater pump with a conveying capacity of 10 $m^3\,h^{-1}$ (ABS Pumpen AG, Lohmar, Germany) and an ultrafiltration unit (ETL Verfahrenstechnik, Peiting, Germany), both as usually applied for wastewater investigations. The ultrafiltration membrane was made of polyethersulfone with a pore width of 40 nm. In this way the pilot plant and the sensor, respectively, were continuously provided with wastewater permeate at a flow rate of 1.5 l h^{-1}. To ensure that the substrate concentration was at a sufficiently high level at any time of the day, ammonium sulphate was added to the testing plant at a concentration of about 60 mg l^{-1} NH_4^+–N.

2.4. Measuring procedure

The sensor is exposed to a surplus of substrate when starting a measurement. The substrate is provided either by a nutrient solution containing 40 mg l^{-1} NH_4^+–N in 0.1 M phosphate buffer or by municipal wastewater, which normally contains NH_4^+–N in the same concentration range. It is also absolutely necessary to provide for oxygen saturation of these media. Under these conditions the basic current or oxygen concentration, respectively, is stabilised at a low level, because the oxygen consumption by the immobilisate increases to the maximum rate and therefore the oxygen molecules diffuse through the bacterial immobilisate in relatively small amounts.

The addition of an inhibitor leads to a rapidly decreasing bacterial oxygen consumption which causes an enhanced oxygen diffusion through the microbial membrane to the probe. This results in increasing currents being registered by the Clark electrode which correspond to increasing oxygen concentrations. When a new steady state is finally reached, this signal is used for quantitation of nitrification inhibiting effects.

3. Results and discussion

3.1. Measurement of wastewater samples with the laboratory device

3.1.1. Response curves

The response curves obtained by examination of wastewater from a refinery, highly enriched with aromatic

compounds, which had an inhibitory effect on a sewage pilot plant at the ISWA are shown in Fig. 1. The inhibitory effect is registered by the sensor after 30 s and a maximum or almost maximum signal is reached within 5.5 min. These response curves are similar to those obtained by measurements with the nitrification inhibitor allyl thiourea (ATU) as described in previous publications (König et al., 1997a, b). ATU is the standard nitrification inhibitor that we used for our investigations and that is also customary for laboratory use as the selective nitrification inhibiting agent when determining biochemical oxygen demand (BOD) (Montgomery and Borne, 1966).

We measured a logarithmic series of sample dilutions (sample dilutions made with the nutrient solution as described in "Measuring procedure") in the range between 1 and 250 ml l^{-1}. The lowest sample dilution step which caused an unambiguous inhibitory signal was 10 ml l^{-1}, whereas at the high dilution step of 250 ml l^{-1} the maximum inhibitory effect (100% inhibition) was observed. The maximum inhibitory effect was confirmed by an investigation with ATU at a concentration of 10 mg l^{-1}. Sensor signals in the range of 2500 nA generally indicate oxygen saturation at the Clark electrode. The inhibitory effect caused by the wastewater sample was reversible for all sample dilutions investigated.

3.1.2. Quantitation of the inhibiting effect

For the relation between the inhibitor concentration (logarithmic scale) and the sensor signal a sigmoidal function was found (Fig. 2). The ΔI observed after 5.5 min was used for quantitation of the inhibiting effect. The sample concentration which caused an inhibitory effect of 50% in comparison with the control investigation (IC_{50}) was 21.1 ml l^{-1}. This means that this refinery wastewater has the same IC_{50} as a solution of ATU with a concentration of 57 mg l^{-1}.

Due to the reversibility of the reaction, the 20 single measurements shown in Fig. 2 could be carried out

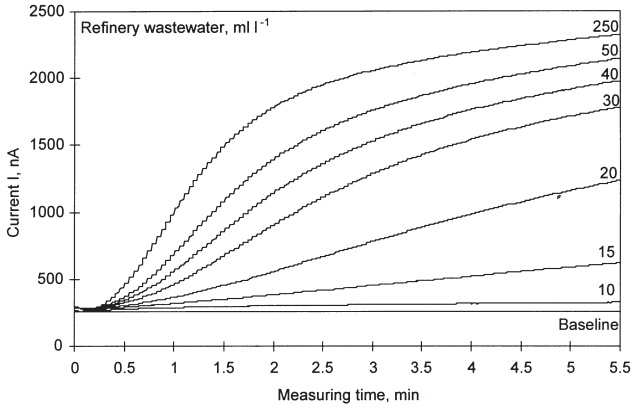

Fig. 1. Response curves of the biosensor for nitrification inhibitors for dosages of refinery wastewater contaminated with several aromatic compounds (signals recorded with the laboratory device).

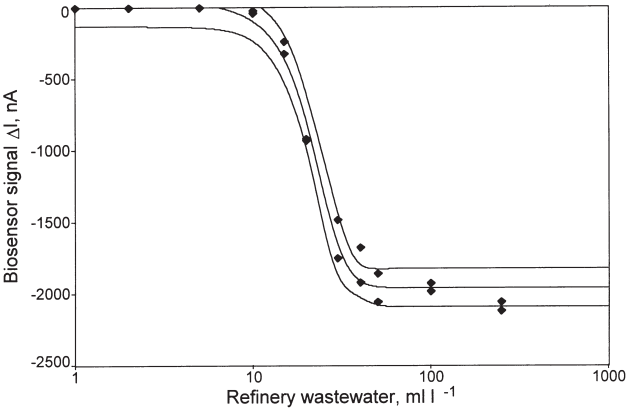

Fig. 2. Concentration dependence of the sensor signal on dosages of the refinery wastewater recorded with the laboratory device. The sensor signals shown in this graph are ΔI-values calculated from the response curves in Fig. 1 (measuring time 5.5 min, $r^2 = 0.9845$, mean deviation from the mean value of two corresponding measurements = 7.3%, confidence interval 99%).

within 2 days using the same sensor membrane. The differences in the measured data shown in this picture are due to the deviation of data normally observed when investigating inhibiting effects rather than to losses of sensitivity by repeated use of the biosensor (see also Fig. 3).

3.1.3. Further laboratory investigations of real samples

The results of investigations of other samples are shown in Table 1. Remarkable is the extremely intensive inhibitory effect caused by commercial grade petrol, probably caused by the high concentration of benzene in this sample (see also Table 2). It exhibited an inhibitory effect as strong as the effect of a solution of ATU with a concentration of 12 g l^{-1}. Petrol also led to a poor reversibility of the sensor activity. The alcohol solution from a fermentation process used as external C-source for denitrification in sewage plants contained unknown impurities. This mixture also proved to be a highly efficient nitrification inhibiting agent. Ethanol itself is known to exhibit a certain inhibitory effect on nitrification (Richardson, 1985). The enzymatic washing agent from the textile industry and a boiling condensate from the meat processing industry represent samples which were not inhibitory at a extremely high degree. Nevertheless the sensor was capable of determining their IC_{50} at relatively high dilution steps of 137 and 180 ml l^{-1}, respectively.

3.1.4. Investigations with industrial chemicals

Several chemicals which have been reported in the literature to act as nitrification inhibitors (Richardson, 1985, 1992), were investigated (Table 2). Generally, a complete concentration series of an inhibiting sample could be recorded within 1 day by the use of one sensor

Table 1
Results determined by the biosensor for nitrification inhibitors for some inhibitory samples. The maximum inhibitory effect and the concentration of an ATU-solution which would cause a 50% inhibitory effect (IC_{50}) at the same dilution is given

Inhibitor	IC_{50} (ml l^{-1})	Equivalents of ATU (mg l^{-1})	Maximum inhibitory effect (%)	Annotations
Petrol	0.11	12 000	95	Commercial quality
Impurities containing alcohol solution from a fermentation process[a]	0.9	1300	100	Waste product of the food industry
Wastewater from a refinery	21	57	85	Pilot sewage plant of the ISWA
Enzymatic washing agent	137	8.8	75	Textile industry
Boiling condensate	180	6.7	100	Meat processing industry

[a]Used as external C-source for denitrification in sewage plants.

Table 2
List of the IC_{50}-values of several substances inhibiting nitrification measured by the laboratory device. All compounds listed caused reversible inhibiting effects

Inhibitor	IC_{50} (mg l^{-1})	IC_{50} (mM)	Maximum inhibitory effect (%)
Allyl thiourea	1.2	0.01	100
Thiourea	0.8	0.01	100
p-Cresol	5.9	0.055	100
Aniline	8.2	0.09	93
Phenol	8.4	0.09	100
Styrene	28.9	0.23	100
p-Xylene	37.5	0.34	85
Benzene	28.4	0.36	100
Acetonitrile	352	0.86	100
Copper[a]	187	2.94	100
Methanol	190	5.94	85
Potassium chlorate[b]	1400	11.4	100

[a]Measuring time 15 min.
[b]Selective inhibitor of nitrite oxidation.

only. This is a very short time compared to the time required for other nitrification inhibiting tests (Wagner and Kayser, 1990; ISO, 1989).

On the basis of molar concentration, we found the strongest inhibition for the selective nitrification inhibitors ATU and thiourea, each with an IC_{50} of 10 μM. For ATU, a detection limit of 1.5 μM and a limit of determination of 2.0 μM was determined. Strong inhibitory effects were also found for phenol, its methyl derivate p-cresol and for aniline. Benzene and its derivates styrene and p-xylene as well as acetonitrile are also strong inhibitors. Copper is an example for the effect of heavy metals and methanol for primary alcohols. Chlorate acts as a selective inhibitor of the second step of nitrification, the oxidation of nitrite to nitrate. There is a distinct inhibitory effect on this nitratation step beginning at concentrations in the 10 mM range.

Under laboratory conditions, the sensor shows a long-term stability of several weeks when using reversible inhibitors as test substances (König et al., 1997a). A long-term investigation with ATU as the nitrification inhibiting agent delivered proof of the sensor stability (Fig. 3). Data were recorded for 1 week for a sensor which had been used for repeated measurements during its lifetime of 6 weeks, and there was no significant loss of signal at the end of these experiments. A reproducibility of at least 95% was calculated (König et al., 1997a).

3.2. Monitoring of wastewater with the field device

3.2.1. Response curves and quantitation

When the pilot plant was operated under the conditions described in "Measuring system", a steady baseline was built up near 0 mg l^{-1} oxygen, indicating that the biosensor for nitrifiers shows full respiratory activity when starting inhibitor tests.

The six peaks shown in Fig. 4 correspond to dosages of thiourea in concentrations of 0.1–10.0 mg l^{-1} into the testing plant influent, lasting 5 min each. It can be seen that the sensor signal is dependent on the concentration of the nitrification inhibitor and that there is a good sensitivity of the sensor under field investigation conditions. After the addition of an inhibitor, it generally takes 2–

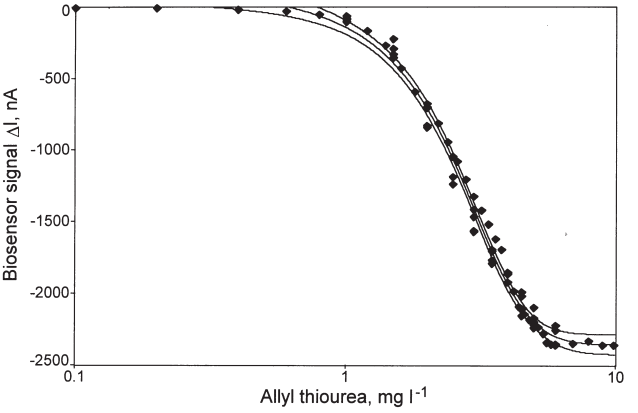

Fig. 3. Long-term stability of the laboratory device tested with allyl thiourea ATU as nitrification inhibiting agent. The sensor lifetime was 6 weeks, the data shown in the graph were recorded within 1 week (measuring time 5.5 min, $r^2 = 0.9845$, coefficient of variation $< 5\%$, confidence interval of 99%).

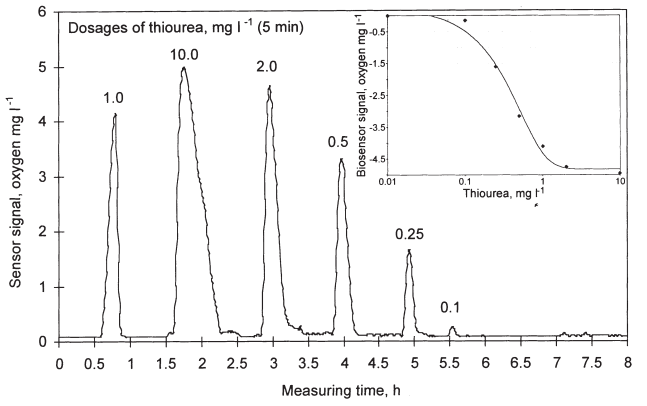

Fig. 4. Typical response curves of the sensor when the inhibitor thiourea is added to the field device for 5 min. The inset demonstrates the influence of the concentration of the inhibitor on the intensity of the sensor signal.

3 mins until the sensor reacts. About 10 min later the signal reaches its maximum value. The regeneration phase following the reaction with the inhibitor is also recorded. The time required for complete regeneration of the biosensor activity took between 15 min and 1 h, depending on the dosage of the inhibitor.

3.2.2. Detection of wastewater streams inhibiting nitrification

During December 1996 to May 1997 the field device was used to continuously monitor a municipal sewage plant. During this time it was possible to detect on-line a wastewater discharge with nitrification inhibiting activity (Fig. 5).

The biosensor signal measured in the influent of the sewage treatment plant rose at about 3 a.m. from a very stable baseline of 0.1 mg l^{-1} O_2 to a value of 1.0 mg l^{-1} O_2, which was reached at 6 a.m. After 6 a.m., the sensor signal decreased and returned to the initial baseline level at 8 a.m.

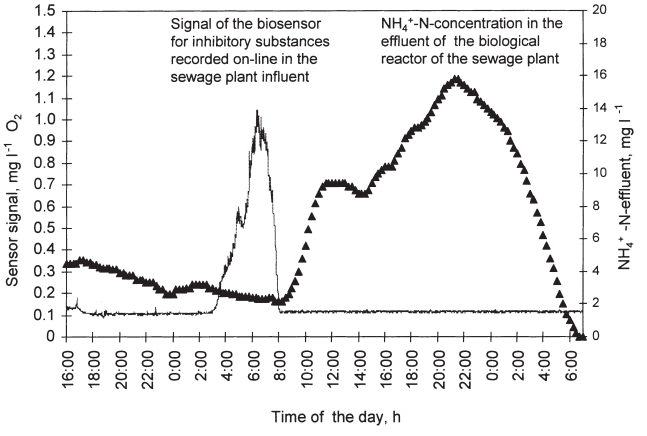

Fig. 5. On-line detection of a pollutant containing wastewater stream with the biosensor at the influent of a municipal sewage plant. The sensor signal correlates in time as well as in intensity with the rising NH_4^+–N concentrations recorded in the effluent of the biological reactor of the sewage plant.

At 9 a.m., i.e. 6 h after the biosensor had begun to register the inhibitor, the ammonium concentration in the effluent of the biological reactor began to increase from 2 mg l^{-1} NH_4^+–N up to a final concentration of 16 mg l^{-1}. Increasing NH_4^+–N concentrations in the effluent typically indicate a disturbance of nitrification. The delay of 6 h between the occurrence of the signal recorded at the influent and the occurrence of the increasing ammonium concentrations corresponded exactly to the time the wastewater needed to flow through the treatment plant.

The intensity of the sensor signal and of the NH_4^+–N concentrations of the effluent can also be correlated. A biosensor signal of 1.0 mg l^{-1} O_2, as shown in Fig. 4, indicates only about 20% of maximum inhibition, equivalent for example to a thiourea dosage of 0.2 mg l^{-1}. According to this, a relatively small inhibitory effect was observed on the sewage plant itself. In the case of a very strong inhibition the NH_4^+–N concentration of the effluent would increase to concentrations in the range of 40–50 mg l^{-1}, which are the normal influent values for municipal wastewater. Thus, the relative low intensity of the biosensor signal correlated favourably with the reaction of the sewage plant.

3.2.3. Long-term stability

A long-term stability for the biosensor of at least 1 week and occasionally up to 3 weeks was achieved, when permanently monitoring raw sewage. This relatively high long-term stability in wastewater was the result of some special construction features of the flow-through cell, where the biosensor was mounted (König et al., 1997b). The intention of all these construction features was to prevent the growth of heterotrophic bacteria on the surface of the sensor membrane and inside the flow-through chamber. The growth of such biofilms is sooner or later inevitable when permanently working with sewage influent (Flemming, 1991) and will reduce the sensitivity of the biosensor. The maintenance effort for the whole field device was about 1 day per week.

4. Conclusions

Both the laboratory and the field device allow a rapid and reproducible detection and quantitation of substances inhibiting nitrification in municipal wastewater. The laboratory device was designed as a simple method for carrying out a quantitation of the nitrification inhibiting potential of a sample. Additionally, the summary quantitation of nitrifiable compounds, the so-called N-BOD, can be measured after slight and simple changes in the measuring procedure (König et al., 1997a, b). When integrated in the field device the biosensor is able to monitor wastewater streams continuously and to

reveal their momentary degree of pollution. This sensor system opens new possibilities for the on-line control of sewage plants.

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